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Rat Cardiac Muscarinic Receptors

I. Effects of Guanine Nucleotides on High- and Low-Affinity Binding Sites

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SUMMARY

The binding properties of muscarinic cholinergic binding sites were investigated in rat cardiac membranes, using the labeled agonist [^{3}H]oxotremorine-M ([^{3}H]Oxo-M) and the labeled antagonist L-[*benzilic*-4,4′-^{3}H]quinuclidinyl benzilate (L-[^{3}H]QNB). The binding of both tracers was inhibited stereospecifically by dexetimide and levitimide. [^{3}H]Oxo-M labeled only high-affinity agonist binding sites, whereas L-[^{3}H]QNB bound to high- and low-affinity agonist binding sites with equal affinity. Agonists were unable to induce "negative cooperativity" interactions by increasing the dissociation of labeled agonist or antagonist. Guanine nucleotides decreased markedly the affinity of high- and low-affinity binding sites for agonists, without affecting their affinity for antagonists. In the presence of a maximally effective concentration of GTP, all muscarinic receptors showed the same low affinity for agonists. Among these agonists, carbamylcholine and oxotremorine (but not pilocarpine) displayed a lower affinity for both classes of binding sites in the presence of GTP.

INTRODUCTION

The binding of agonists to muscarinic cholinergic receptors from cardiac muscle (1–5) and other tissues (6–9) does not follow the law of mass action for a single class of receptors. Birdsall *et al.* (8) have demonstrated the existence of two major populations of agonist binding sites in rat cortex with the same affinity for antagonists (10) but different affinities for agonists. Agonists are clearly unable to induce the interconversion of both types of muscarinic receptors (8). Guanine nucleotides decrease the affinity of cholinergic agonists for cardiac (11–15) and brain (16, 17) muscarinic receptors, without affecting markedly the affinity of antagonists (11–17).

In this work, we compared the binding properties, at 25°, of the labeled agonist [^{3}H]Oxo-M[1] and of the labeled antagonist L-[^{3}H]QNB to rat heart membranes. This allowed a differentiation and a direct determination of the concentration of agonist binding sites with high- and low-affinity, respectively. The effects of guanine nucleotides on the affinities of both populations of binding sites were examined.

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[1] The abbreviations used are: [^{3}H]Oxo-M, [*methyl*-^{3}H]oxotremorine acetate; L-[^{3}H]QNB, L-[*benzilic*-4,4′-^{3}H]quinuclidinyl benzilate; p(NH)ppG, guanosine 5′-*O*-(2,3-imido)triphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate.

MATERIALS AND METHODS

Chemicals

L-[^{3}H]QNB (specific radioactivity 40 Ci/mmole) and [^{3}H]Oxo-M (specific radioactivity 84 Ci/mmole) were obtained from New England Nuclear Corporation (Dreieich, Federal Republic of Germany). Oxotremorine sesquifumarate was obtained from Aldrich Chemical Company (Beerse, Belgium); atropine from Sigma Chemical Company (St. Louis, Mo.); and pilocarpine and carbamylcholine from Federa (Brussels, Belgium). Dexetimide and levitimide were generous gifts from Janssen Pharmaceutica (Beerse, Belgium). Nucleotides were purchased from Boehringer (Mannheim, Federal Republic of Germany).

Methods

Preparation of cardiac membranes. Rat cardiac membranes were prepared according to the procedure of Snyder and Drummond (18), with a few modifications. Male albino Wistar rats (200–250 g), fed rat chow ad libitum, were killed by decapitation. The hearts were quickly removed, rinsed at room temperature with isotonic sodium chloride, and stored in liquid nitrogen. All subsequent operations were performed at 4°. Each heart was homogenized with a glass-Teflon homogenizer in 5 ml of 20 mM Tris-HCl (pH 7.5) enriched with 2 mM β-mercaptoethanol and 5 mM $MgCl_2$. After a 2-fold dilution with the same buffer, the homogenate was filtered

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through two layers of medical gauze and centrifuged at 520 × *g* for 10 min. The resulting pellet was washed once with 10 ml of homogenization buffer. The pelleted membranes were resuspended in 5 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 5 mM $MgCl_2$. An equal volume of 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose, 2 mM β-mercaptoethanol, and 2.5 M KCl was added dropwise. This mixture was left for 1 hr in an ice bath, under continuous stirring, then centrifuged at 37,000 × *g* for 10 min. The resulting pellet was washed three times in 5 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 2 mM β-mercaptoethanol. The membranes were finally resuspended in the last buffer, at a concentration of approximately 6 mg of protein per milliliter, and stored in liquid nitrogen. Protein concentrations were determined by the method of Lowry *et al.* (19).

Binding assays. Heart membrane proteins (0.13 mg/ml) were incubated at 25° in 1.2 ml of 50 mM sodium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$, 1% bovine serum albumin, and the indicated concentrations of L-[^{3}H]QNB or [^{3}H]Oxo-M, unlabeled agonist, and unlabeled antagonist. Specific binding was measured as follows. Each sample was filtered on glass-fiber filters GF/C (Whatman, Maidstone, England) and rinsed four times with 2 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.5) enriched with 0.25 M sucrose, 2 mM β-mercaptoethanol, and 1% bovine serum albumin. The total contact time between the filters and rinsing buffer was less than 8 sec, so that dissociation of the high-affinity [^{3}H]Oxo-M complex described in this paper was probably negligible (half-life of the complex >30 min at 5°). It is of course possible that [^{3}H]Oxo-M binding to a second, rapidly dissociating class of receptors was lost during the filtration procedure. The filters were dried and the radioactivity was counted by liquid scintillation. Nonspecific binding was defined as tracer binding in the presence of 1 μM atropine. This radioactivity represented no more than 10% of the total L-[^{3}H]QNB bound, and 20–30% of the total [^{3}H]Oxo-M bound at equilibrium, and was subtracted from total binding, yielding specific binding.

1. Association kinetics. Rat heart membranes were incubated in the presence of 0.6 nM L-[^{3}H]QNB or 0.3 nM [^{3}H]Oxo-M and in the presence or absence of 1 μM atropine. Specific binding was determined as above, at appropriate time intervals.

2. Dissociation kinetics. Rat heart membranes were preincubated for 10 min at 25° in 1.2 ml of standard medium in the presence of 0.6 nM L-[^{3}H]QNB or 0.3 nM [^{3}H]Oxo-M and in the presence or absence of atropine. Aliquots containing 12 μl of water (control), or atropine (allowing a final concentration of 1 μM), oxotremorine (final concentration 100 μM), carbamylcholine (final concentration 100 μM), or atropine and GTP (final concentration 1 μM and 10 μM, respectively) were then added. After appropriate time intervals, duplicate samples were filtered as indicated above. Results were expressed as percentage of control binding.

3. Scatchard plots. Heart membranes were incubated as indicated above in the presence of increasing concentrations of L-[^{3}H]QNB (0.1–3 nM) or [^{3}H]Oxo-M (0.1–3 nM) and in the presence or absence of 1 μM atropine. Specific binding was measured as indicated above after 10-min or 60-min incubation periods with L-[^{3}H]QNB and 10-min incubation periods with [^{3}H]Oxo-M. The results were analyzed according to the method of Scatchard (20).

4. Competition curves. Heart membranes were incubated for 10 min as indicated above in the presence of a fixed concentration of L-[^{3}H]QNB (0.6 nM) or [^{3}H]Oxo-M (0.3 nM) and the indicated concentrations of agonists or antagonists. Specific binding was measured as above, after 10 min with L-[^{3}H]QNB and [^{3}H]Oxo-M or 1 hr with L-[^{3}H]QNB. Under these conditions, maximal binding represented 5% (L-[^{3}H]QNB) or 1% ([^{3}H]Oxo-M) of the total radioactivity added. Competition curves with L-[^{3}H]QNB were analyzed by computer fitting to a model of two classes of binding sites (21). The dissociation constants of the analogues tested (using both tracers) were corrected according to the method of Cheng and Prusoff (22).

RESULTS

L-[^{3}H]QNB binding sites and [^{3}H]Oxo-M binding sites were compared on the basis of association and dissociation kinetics, Scatchard plots at equilibrium, competition with antagonists and agonists, and the effects of guanine nucleotides on these parameters.

Association Kinetics

L-[^{3}H]QNB binding at 25° reached the steady state after 1 hr of incubation when tested at a low concentration (0.1 nM) of ligand (data not shown), whereas it reached the steady state after only 10 min (Fig. 1) when tested at the higher concentration (0.6 nM) that was used for routine competition curves (see below). [^{3}H]Oxo-M binding reached the steady state within 10 min of incubation at all concentrations investigated (0.1–3 nM; Fig. 1 and data not shown). The association rate constants

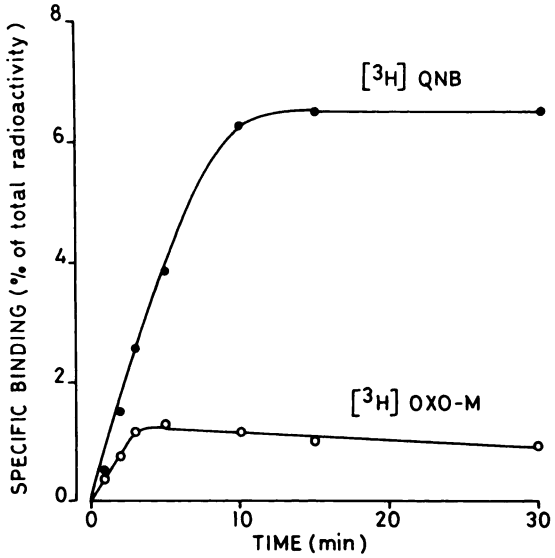

FIG. 1. *Time course of L-[^{3}H]QNB (0.6 nM) and [^{3}H]Oxo-M (0.3 nM) binding to rat heart membranes at 25°*

Specific binding was determined as a function of time as indicated under Methods. This experiment is typical of four others.

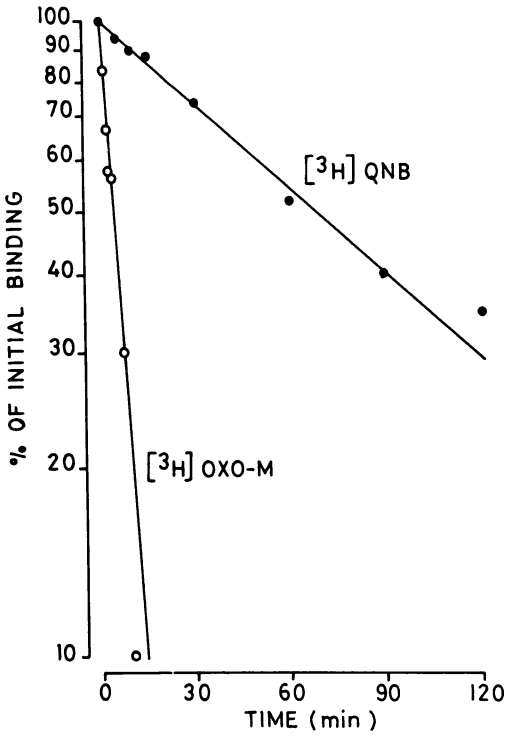

FIG. 2. *Time course of dissociation of L-[^{3}H]QNB (0.6 nM) and [^{3}H]Oxo-M (0.3 nM) from rat heart membranes at 25°*

Specific binding was determined as a function of time as indicated under Methods. The results were expressed as a percentage of "control" observed after addition of water only (see Methods). This experiment is typical of three others.

were approximately 1.5 10^8 M/min ([^{3}H]QNB) and 1.1 10^8 M/min ([^{3}H]Oxo-M), respectively.

Dissociation Kinetics

L-[^{3}H]QNB dissociated slowly from rat heart membranes at 25° in the presence of 1 μM atropine, 100 μM oxotremorine, or 100 μM carbamylcholine. The half-life of the complex was 76 ± 15 min (n = 4; Fig. 2) (dissociation rate constant 9.1 10^{-3} min^{-1}). [^{3}H]Oxo-M dissociated much more rapidly under the same conditions, the half-life of the complex being only 3.3 ± 0.9 min (n = 4) (Fig. 2) (dissociation rate constant 0.21 min^{-1}).

Scatchard Plots

After 10 min of incubation, L-[^{3}H]QNB bound with a low apparent dissociation constant (K_d = 0.60 ± 0.03 nM, n = 5) to rat heart membranes. There was no evidence for heterogeneity among muscarinic antagonist binding sites (Fig. 3), and the receptor concentration was 279 ± 48 fmoles/mg of protein. The true dissociation constant, measured after 1 hr of incubation at 25°, was lower (0.10 ± 0.02 nM, n = 3) [in good agreement with the value calculated from the association and dissociation rate constants (0.06 nM)] and, under these conditions, the total receptor concentration was somewhat lower (by 10–20%) than that observed after 10 min in parallel incubations (data not shown).

[^{3}H]Oxo-M bound with a higher dissociation constant (K_d = 2.0 ± 0.1 nM, n = 5) to rat heart membranes. This K_d value was in good agreement with the K_d value calculated from the association and dissociation rate constants (1.9 nM). There was no evidence for heterogeneity among agonist binding sites (Fig. 3). However, because of relatively high nonspecific binding, the existence of a second class of low-affinity binding sites could not be excluded. The receptor concentration was 127 ± 17 fmoles/mg of protein, i.e., 47.5 ± 5.3% of the receptor concentration measured by L-[^{3}H]QNB binding in parallel incubations. Preincubation of the membranes for 50 min at 25° provoked a 20–40% decrease in the number of [^{3}H]Oxo-M binding sites, indicating that high-affinity binding sites were not stable during prolonged incubation. Therefore, a short incubation period (10 min) was

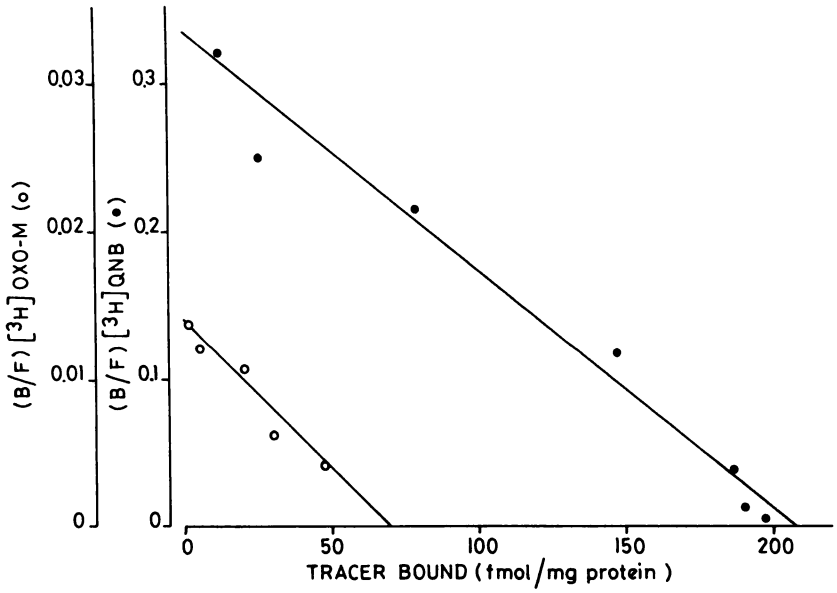

FIG. 3. *Scatchard plots of L-[^{3}H]QNB (●) (0.1–3.0 nM) and [^{3}H]Oxo-M (○) (0.1–2.0 nM) binding to rat heart membranes*

Specific binding was measured after 10 min of incubation at 25°, as indicated under Methods. This experiment is typical of four others.

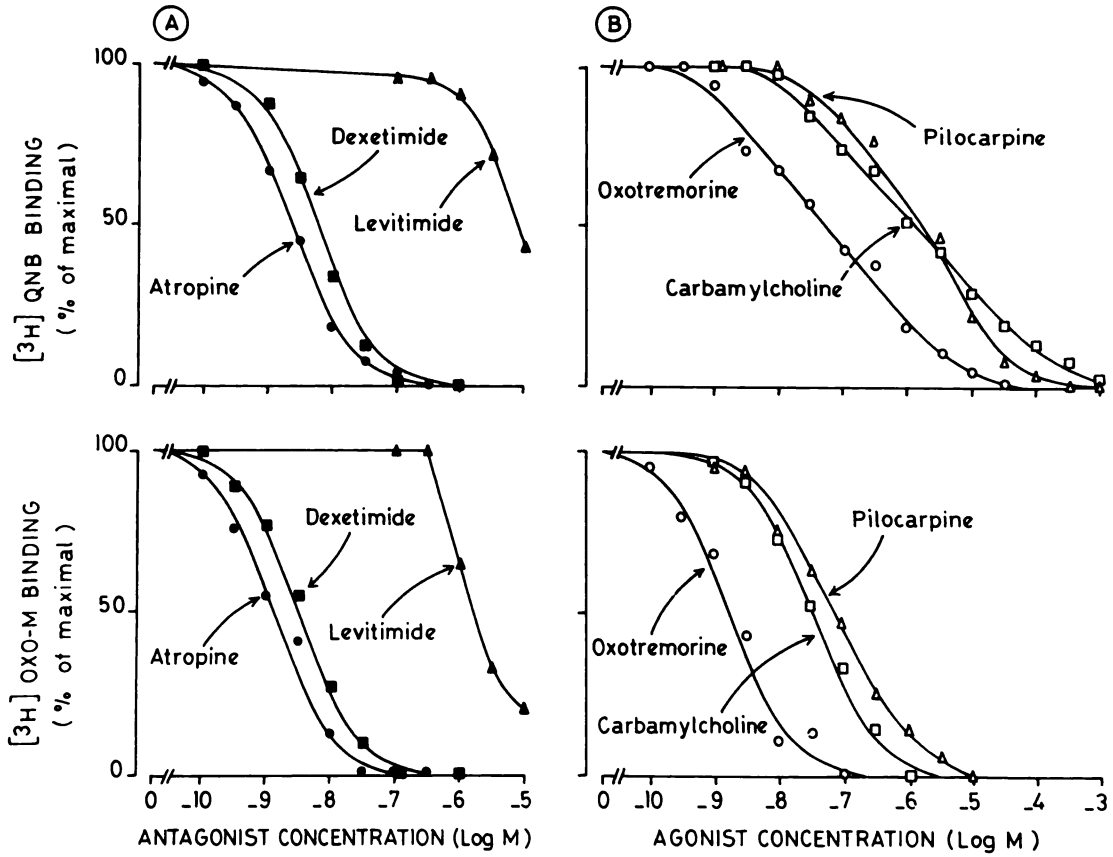

FIG. 4. *Dose-effect curves of inhibition of L-[^{3}H]QNB and [^{3}H]Oxo-M binding to rat heart membranes by three muscarinic antagonists (A) and three muscarinic agonists (B)*

Rat heart membranes (0.13 mg/ml) were incubated for 10 min at 25° in the presence of 0.6 nM L-[^{3}H]QNB or 0.3 nM [^{3}H]Oxo-M and the indicated concentrations of atropine, dexetimide, levitimide, oxotremorine, carbamylcholine, or pilocarpine. Specific binding was measured as indicated under Methods. The results were expressed as percentage of specific binding in the absence of unlabeled analogue. This experiment is representative of three to ten experiments.

used to measure the relative concentration of sites binding [^{3}H]Oxo-M and L-[^{3}H]QNB (see below).

Competition Curves

L-[^{3}H]QNB was used at a high concentration (0.6 nM) so that the steady state was reached within 10 min (Fig. 1). The dissociation constants, calculated according to the method of Cheng and Prusoff (22), were identical after 10 min or 1 hr of incubation for all unlabeled antagonists and agonists tested (data not shown).

Antagonists. Levitimide [the inactive stereoisomer of dexetimide (23)] decreased L-[^{3}H]QNB (0.6 nM) and [^{3}H]Oxo-M (0.3 nM) binding at concentrations 300–1000 times higher than those of dexetimide (the active stereoisomer), suggesting that both tracers were displaced stereospecifically by antagonists (Fig. 4A). Competition

TABLE 1

Equilibrium dissociation constants of two antagonists and three agonists binding to muscarinic receptors in rat heart membranes labeled with L-[^{3}H]QNB in the absence and presence of GTP

Competition curves for L-[^{3}H]QNB were fitted to a model of two classes of binding sites according to ref. 21. The concentration of L-[^{3}H]QNB used in these experiments varied between 0.45 and 0.75 nM. The K_d values for both classes of binding sites were calculated according to the method of Cheng and Prusoff (22). In the presence of GTP, the fraction of high-affinity binding sites was not significant (3 ± 4, 11 ± 10, and 0 ± 4% of the receptors, using oxotremorine, carbamylcholine, and pilocarpine, respectively). Results are means ± standard deviation of four to six experiments.

Analogue	Fraction of high-affinity binding sites	High-affinity binding sites	Low-affinity binding sites	Binding sites in presence of 10 μM GTP
	%	M	M	M
Atropine		$0.9 \pm 0.5\ 10^{-9}$	$0.9 \pm 0.5\ 10^{-9}$	$1.1 \pm 0.4\ 10^{-9}$
Dexetimide		$1.5 \pm 0.2\ 10^{-9}$	$1.5 \pm 0.2\ 10^{-9}$	ND[a]
Oxotremorine	49 ± 16	$2.7 \pm 1.9\ 10^{-9}$	$2.9 \pm 2.2\ 10^{-7}$	$9.6 \pm 2.7\ 10^{-7}$
Pilocarpine	23 ± 14	$3.7 \pm 2.6\ 10^{-8}$	$2.6 \pm 1.2\ 10^{-6}$	$4.3 \pm 0.7\ 10^{-6}$
Carbamylcholine	37 ± 10	$3.8 \pm 2.9\ 10^{-8}$	$3.7 \pm 2.7\ 10^{-6}$	$4.3 \pm 1.9\ 10^{-5}$

[a] ND, Not detectable.

TABLE 2

Equilibrium dissociation constants of two antagonists and three agonists binding to high-affinity muscarinic binding sites labeled with [^{3}H]OXO-M

Results are means ± standard deviation of four experiments.

Analogue	K_d
	M
Atropine	$1.2 \pm 0.4\ 10^{-9}$
Dexetimide	$3.2 \pm 1.2\ 10^{-9}$
Oxotremorine	$1.8 \pm 0.6\ 10^{-9}$
Pilocarpine	$1.1 \pm 0.4\ 10^{-7}$
Carbamylcholine	$2.8 \pm 1.8\ 10^{-8}$

curves with the two unlabeled antagonists atropine and dexetimide indicated that both tracers bound to a single class of receptors. The K_d values for atropine and dexetimide, after correction for tracer concentration (22), were identical using both tracers (Tables 1 and 2).

Agonists. Competition curves with the three unlabeled agonists oxotremorine, carbamylcholine, and pilocarpine (Fig. 4B) indicated that L-[^{3}H]QNB bound either to two distinct classes of receptors with different affinities for agonists or to one class of receptors, on which agonists induced negative cooperativity interactions (24). Indeed, the Hill coefficient was lower than 1.0 in all cases (0.59 ± 0.09 with oxotremorine, 0.49 ± 0.07 with carbamylcholine, and 0.75 ± 0.10 with pilocarpine). As agonists did not increase the dissociation rate of L-[^{3}H]QNB as compared with atropine (Fig. 2), we analyzed the results according to a model of two classes of sites (21). The dissociation rate constants, corrected according to the method of Cheng and Prusoff (22), are indicated in Table 1.

The relative concentration of high-affinity binding sites, derived from competition curves of the two full agonists (37 ± 10% for carbamylcholine; 49 ± 16% for oxotremorine) (Table 1) was similar to, but less precise than, that measured with Scatchard plots of [^{3}H]Oxo-M.

[^{3}H]Oxo-M recognized a single class of agonist binding sites (Fig. 4B). The K_d values of agonists for this receptor [corrected according to the method of Cheng and Prusoff (22)] were identical with those of the "high-affinity receptors" identified by competition curves for L-[^{3}H]QNB (Table 2).

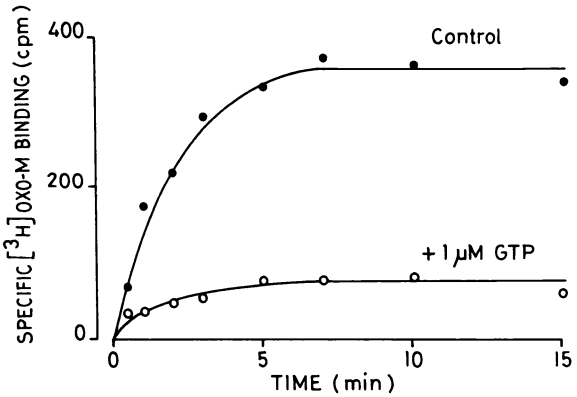

FIG. 5. *Time course of [^{3}H]Oxo-M (0.3 nM) binding to rat heart membranes at 25° in the absence and presence of 1 μM GTP*

Specific binding was measured as a function of time as indicated under Methods. This experiment is typical of two others.

Effects of Guanine Nucleotides on Agonist Binding

Association kinetics. GTP did not affect L-[^{3}H]QNB binding (results not shown). At a submaximal concentration (1 μM), GTP markedly decreased [^{3}H]Oxo-M binding. This rapid effect was maintained for at least 15 min of incubation (Fig. 5). [^{3}H]Oxo-M binding in the presence of 10 μM GTP was not detectable (Fig. 8).

Dissociation kinetics. GTP at a 10 μM concentration did not affect the dissociation rate of L-[^{3}H]QNB as measured by addition of an excess of unlabeled atropine (data not shown). By contrast, the dissociation rate of [^{3}H]Oxo-M increased so markedly in the presence of GTP (Fig. 6) that the half-life of the complex could not be measured owing to very low residual binding after 20 sec of dissociation.

Equilibrium binding. GTP (10 μM) was without effect on the total number and K_d value of L-[^{3}H]QNB receptors and on competition curves with atropine (Table 1 and data not shown). By contrast, competition curves obtained with oxotremorine, carbamylcholine, and pilocarpine were markedly modified in the presence of GTP (Fig. 7 and Table 1). The Hill coefficients were significantly increased in the presence of GTP (0.87 ± 0.04 for oxotremorine, 0.84 ± 0.04 for carbamylcholine, and 1.06 ± 0.15 for pilocarpine). The fraction of high-affinity binding sites was not significant under these conditions

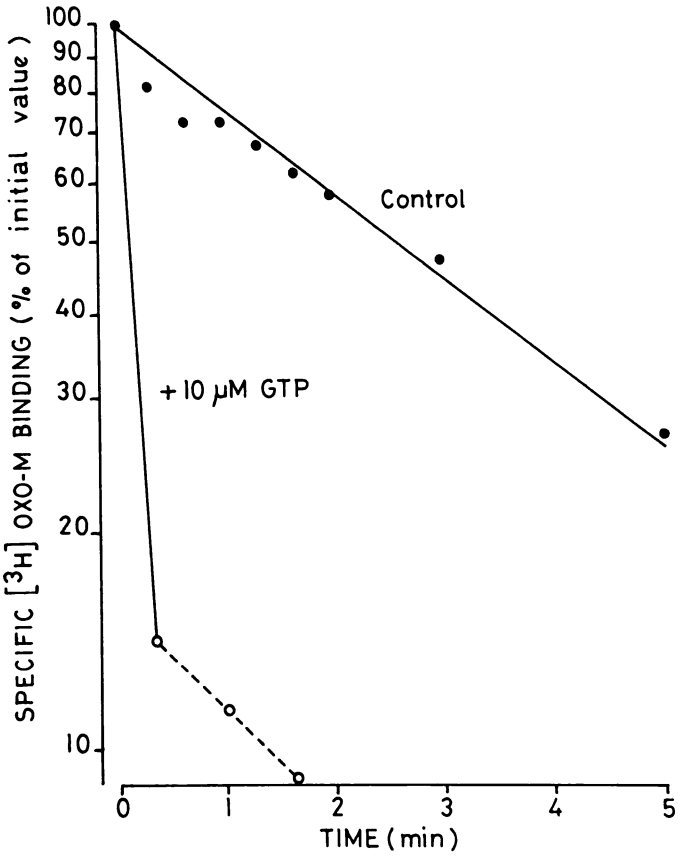

FIG. 6. *Time course of dissociation of [^{3}H]Oxo-M from rat heart membranes in the absence and presence of 10 μM GTP*

Specific binding was determined as a function of time, as indicated under Methods. The results were expressed as a percentage of control specific binding (see Methods). This experiment is typical of two others.

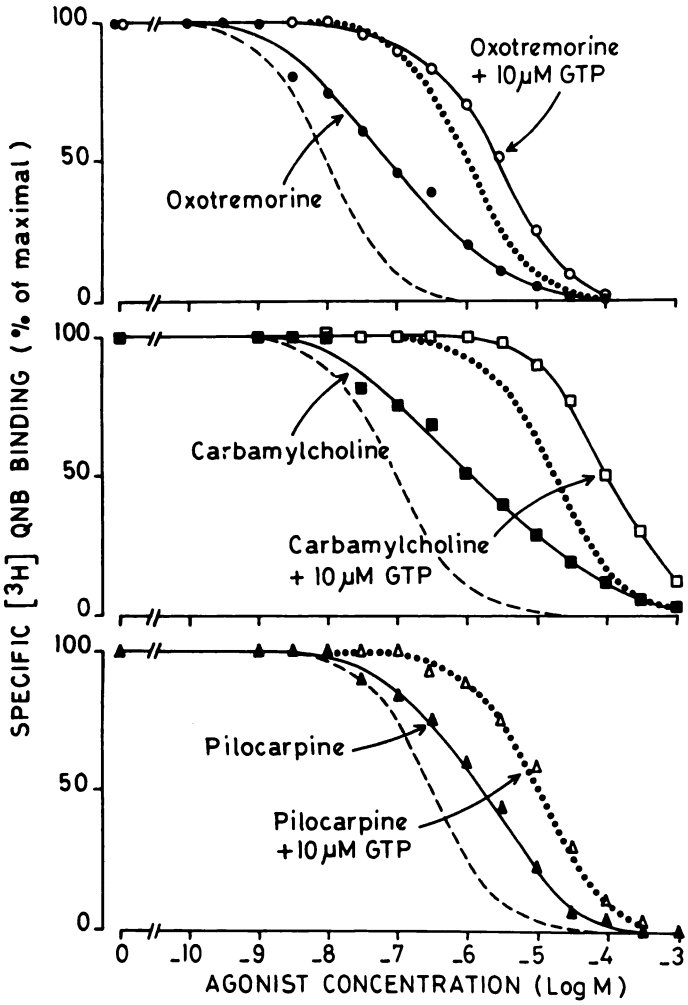

FIG. 7. *Effects of GTP on dose-effect curves of inhibition of L-[^{3}H]QNB binding to rat heart membranes by three muscarinic agonists*

Rat heart membranes (0.13 mg/ml) were incubated for 10 min at 25° in the presence of 0.6 nM L-[^{3}H]QNB and the indicated concentrations of oxotremorine, carbamylcholine, or pilocarpine, and in the presence (*open symbols*) or absence (*closed symbols*) of 10 μM GTP. Specific binding was measured as indicated under Methods. The results were fitted assuming that 50% of the receptors had a high affinity for agonists (EC_{50} 10^{-8} M, 10^{-7} M, and 3.10^{-7} M for oxotremorine, carbamylcholine, and pilocarpine) (– – –) and 50% of the receptors had a low affinity for agonists (EC_{50} 10^{-6} M, 2.10^{-5} M, and 10^{-5} M for oxotremorine, carbamylcholine, and pilocarpine (····). This experiment is representative of four to six experiments.

(see legend to Fig. 2). These competition curves were therefore analyzed, assuming a single class of very low-affinity binding sites. The K_d of pilocarpine in the presence of GTP was similar to that of low-affinity binding sites in the absence of GTP. This was not the case for oxotremorine and carbamylcholine; their K_d values were lower in the presence than in the absence of GTP (Table 1).

Dose-response curves for guanine nucleotide inhibition of [^{3}H]Oxo-M binding. The three guanine nucleotides GTP, p(NH)ppG, and GTPγS completely inhibited [^{3}H]Oxo-M binding to rat heart membranes (Fig. 8), and increased the IC_{50} of carbamylcholine for L-[^{3}H]QNB-labeled receptors to the same extent (25).

GTPγS was the most potent nucleotide in both systems (IC_{50} 20 nM), followed by GTP (IC_{50} 50 nM) and p(NH)ppG (IC_{50} 100 nM) (Fig. 8 and data not shown).

DISCUSSION

The binding properties of the labeled agonist [^{3}H]Oxo-M and of the labeled antagonist L-[^{3}H]QNB to rat heart membranes were compared at 25°, in the absence and presence of guanine nucleotides.

Both tracers bound reversibly to rat heart membranes. [^{3}H]Oxo-M binding was lower than L-[^{3}H]QNB binding, in part because of the greater dissociation rate of [^{3}H]Oxo-M (Figs. 1 and 2).

The antagonist L-[^{3}H]QNB recognized a single class of binding sites with a high affinity (K_d = 0.1 nM) (Fig. 3). As shown previously (1–5), competition curves of L-[^{3}H]QNB with agonists developed on more than 2 logarithms (Fig. 4B). Several mechanisms could account for these flat competition curves: the agonists could recognize two or more classes of binding sites with different affinities, induce "negative cooperativity" interactions between binding sites (24), or modify the affinity of the receptors as a result of effector coupling (26). The last two models were tested (Fig. 2) by comparing dissociation rates in the presence of an excess of unlabeled analogues that were putative "cooperative" and "noncooperative" analogues (i.e., agonists and antagonists, respectively), assuming that high occupancy should provoke a greater dissociation rate (24, 26). The dissociation rate constants of L-[^{3}H]QNB and [^{3}H]Oxo-M induced by competition

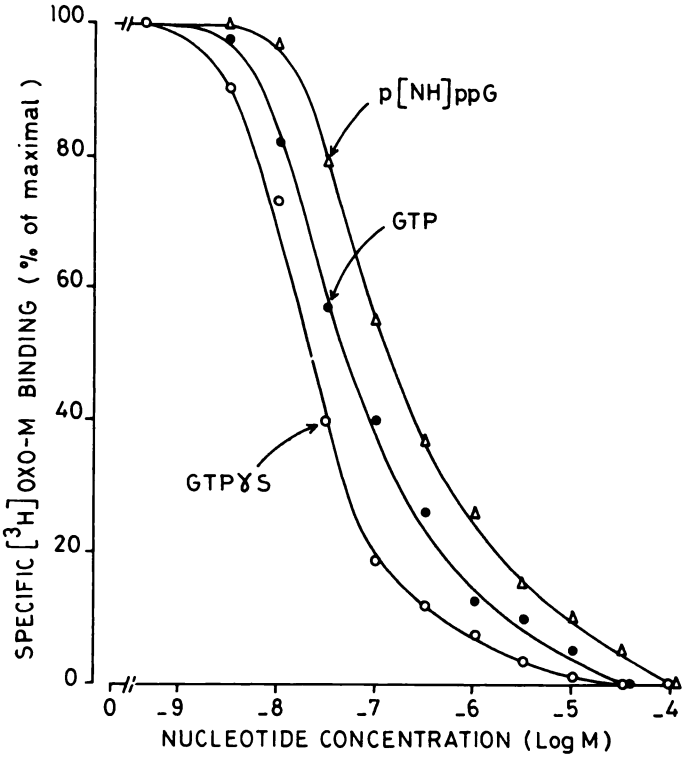

FIG. 8. *Dose-effect curves of inhibition of [^{3}H]Oxo-M binding to rat heart membranes by three guanine nucleotides*

Rat heart membranes (0.13 mg/ml) were incubated for 10 min at 25° in the presence of 0.3 nM [^{3}H]Oxo-M and the indicated concentrations of GTPγS, GTP, or p(NH)ppG. Specific binding was measured as indicated under Methods. Results represent the means of three experiments and are expressed as percentage of specific binding observed in the absence of guanine nucleotides.

with an excess of unlabeled atropine on the one hand (giving "normal" competition curves; Fig. 4) and of carbamylcholine or oxotremorine on the other hand (giving "flat" competition curves; Fig. 4) were identical. Therefore, we analyzed agonist-L-[^{3}H]QNB competition curves according to a "two classes of binding sites" model (21) (see below).

The agonist [^{3}H]Oxo-M apparently recognized a single class of binding sites. The concentration (determined by Scatchard analysis of [^{3}H]Oxo-M binding) and specificity (deduced from competition curves) of [^{3}H]Oxo-M binding sites corresponded exactly to those of "high-affinity agonist binding sites" labeled with L-[^{3}H]QNB. Binding of [^{3}H]Oxo-M to "low-affinity agonist binding sites" was too low to be measured accurately because of high nonspecific binding, and probably represented no more than 10% of total binding. [^{3}H]Oxo-M binding thus allowed an accurate determination of the concentration of high-affinity binding sites.

Guanine nucleotides have been shown to inhibit strongly agonist binding to muscarinic receptors (11–17). We assumed that our heart membrane preparations were virtually devoid of endogenous GTP since (a) hormone stimulation of adenylate cyclase activity was totally dependent on added GTP (27) and (b) preincubation of the membranes with GMP in the presence of isoproterenol, followed by careful washing [a procedure thought to eliminate bound GDP and GTP (28)], had no effect on carbamylcholine binding or on the inhibition of agonist binding by guanine nucleotides (data not shown). We therefore studied the effects of GTP and of the two nonhydrolyzable analogues p(NH)ppG and GTPγS on muscarinic receptors in this system.

At a submaximal GTP concentration (1 μM), [^{3}H]Oxo-M binding was markedly reduced (Fig. 5). This effect was rapid, was stable for at least 15 min at 25°, and was mediated at least in part by a large increase of the dissociation rate constant of [^{3}H]Oxo-M (Fig. 6).

[^{3}H]Oxo-M binding was totally inhibited in the presence of 10 μM GTPγS, 30 μM GTP, and 100 μM p(NH)ppG (Fig. 8). By contrast, L-[^{3}H]QNB binding was unaffected by guanine nucleotides.

Competition curves between L-[^{3}H]QNB and oxotremorine, carbamylcholine, or pilocarpine for binding were markedly affected by GTP (Fig. 7). The three muscarinic agonists recognized a single class of very low-affinity binding sites in the presence of GTP (Hill coefficients of 0.87 ± 0.04, 0.84 ± 0.04, and 1.06 ± 0.15, respectively, for oxotremorine, carbamylcholine, and pilocarpine). These results contradict previous studies on L-[^{3}H]QNB binding to rat heart membranes (11–15) showing that p(NH)ppG decreased the affinity of agonists for muscarinic receptors without affecting their Hill coefficients. Preliminary experiments have shown that this discrepancy may be explained by the temperature chosen—25° in our study (i.e., a temperature favoring receptor stability) rather than 30° (11) or 37° (12–17).

The specificity of binding sites in the presence of GTP was not identical with that of high- and low-affinity binding sites in the absence of GTP; Indeed, carbamylcholine and oxotremorine showed a lower affinity for both classes of binding sites in the presence of GTP than for low-affinity binding sites in the absence of GTP whereas pilocarpine showed the same affinity for low-affinity binding sites in the presence and absence of GTP.

In conclusion, two classes of binding sites having the same affinity for antagonists were characterized in rat heart membranes. The use of two tracers, L-[^{3}H]QNB (binding with the same affinity to both classes of binding sites) and [^{3}H]Oxo-M (binding only to high-affinity binding sites), allowed an accurate determination of the concentration of the two binding sites. Guanine nucleotides markedly inhibited agonist binding to both classes of binding sites. We were unable to demonstrate an interconversion between the two classes of sites. Indeed, (a) agonists did not induce "negative cooperativity" interactions between binding sites, and (b) the specificity of muscarinic receptors in the presence of GTP was different from that observed in the absence of GTP.

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